AD_____

GRANT NUMBER DAMD17-96-1-6025

TITLE: Novel Antiangiogenic/Cytotoxic Therapies for Advanced Breast Cancer

PRINCIPAL INVESTIGATOR: James W. Fett, Ph.D.

CONTRACTING ORGANIZATION: Harvard Medical School
Boston, Massachusetts 02115

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE Novel Antiangiogenic/Cytotoxic Therapies for Advanced Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6025

6. AUTHOR(S)
James W. Fett, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Harvard Medical School
Boston, Massachusetts 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200* It is now well documented that angiogenesis is required for the growth of both primary and metastatic breast cancer. Hence, inhibition of tumor-associated blood vessel growth should be an effective means of treating this disease. Our laboratory has investigated the structure/function relationships of a potent angiogenic mediator, termed angiogenin (Ang). Several inhibitors of its functions have been produced by us and shown previously to possess antitumor activity in preclinical models against a range of human solid tumor types. On this basis we were awarded a grant from the Department of Defense to extend our studies to the treatment of breast cancer. During the first year of funding we have developed reproducible models in athymic mice for the growth and metastatic spread of human breast caner cell lines. We have shown that an antisense drug that targets Ang and an antiAng monoclonal antibody (mAb) inhibit establishment of estrogen-dependent and independent tumor cell lines in mice. A chimeric analogue of this mAb has been constructed for eventual clinical use and its humanization is underway. We have also demonstrated that Ang is present in surgically-excised breast tumor tissue, an important observation for determining patient populations that should benefit from antiAng therapy. Thus, we continue to validate our contention that targeting Ang should be a novel means of combating breast carcinoma.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

JWF In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JWF In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JWF In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JWF In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

James W. Fett 9/26/97

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Background. Carcinoma of the breast is now the most commonly diagnosed cancer among women in the United States and is second only to lung cancer in female cancer-related deaths. It is estimated that over 180,000 new cases will be diagnosed and approximately 44,000 individuals will die from this disease during 1997 (1). While local noninvasive breast cancer in many cases is curable, a sizeable proportion of these patients experience relapse. Furthermore, metastatic disease to distant sites is at present generally considered incurable with treatment aimed at increasing survival while maintaining a reasonable quality of life. Thus, more effective therapeutic maneuvers for the treatment or prevention of advanced, metastatic breast cancer are urgently needed.

Ideally, therapeutic goals for those patients with advanced breast cancer include prevention of development of metastasis where it has not occurred and eradicating occult or detectable metastatic lesions already present - both in conjunction with eliminating the primary cancer. Alternatively, in cases where complete cure or prevention of metastasis is not possible, managing patients in a state of metastatic "stasis" would also be a desirable outcome. We, as others, believe that novel therapeutic strategies that include an *antiangiogenic* component offer realistic hope of achieving these goals in the near future.

The critical contribution of angiogenesis - the development of a hemovascular network - to the growth of solid tumors is now undisputed (2). Angiogenesis also is a prerequisite for the development of metastasis since it provides the means whereby tumor cells disseminate from the original primary tumor, traverse through the circulation and establish at distant sites (reviewed in ref. 3). Therefore, interference with the process of tumor-induced angiogenesis should be an effective therapy for both primary and metastatic cancers. Our own work along with the recent reports that drugs (4) as well as antibodies to a secreted angiogenesis factor (5) suppress the growth and metastatic spread of tumors in animals now lend solid experimental support to this proposition. Indeed, at least nine antiangiogenic agents are in clinical trials for treatment of several angiogenesis-dependent diseases, including cancer (M. J. Folkman, personal communication).

Importantly, the degree of angiogenesis in early-stage breast cancer correlates positively with metastatic recurrence and survival (6). Hence, interference with the angiogenic process in breast cancer should have profound therapeutic consequences.

Angiogenin. The recognized importance of neovascularization in cancer has prompted intensive efforts toward identifying the chemical mediators involved in this process. We have since 1985 studied the structure/function relationships of a potent tumor-associated mediator of the angiogenic process that we named angiogenin (Ang) (7). It has subsequently been extensively characterized both structurally and functionally. The 3-dimensional structure of Ang is now known (8) and is providing a basis for rational design of potential therapeutic inhibitors.

Several antagonists of Ang's activity are available to us and are being evaluated as potential cancer therapeutics. Since when we initiated these studies in the mid-1980's reproducible models for the study of human tumor metastasis in mice were not available, we developed a subcutaneous (sc) tumor model in athymic mice which mimics metastasis to investigate the effect of Ang antagonists on the establishment and growth of human tumor xenografts. This model (termed sc prophylactic), in which a small number of tumor cells are injected sc, approximates in its early stage metastatic disease where a limited number of tumor cells "seed" to a site and establish and grow only if, among other criteria, they receive an adequate supply of blood vessels from the host. In initial studies we demonstrated that prophylactic treatment with the neutralizing antiAng monoclonal antibody (mAb) 26-2F (the antibody is described in ref. 9), which is not cytotoxic to tumor cells *in vitro*, in all cases delayed the appearance of HT-29 colon adenocarcinoma tumors and, strikingly,

completely prevented their establishment in 25% of the mice (10). In subsequent studies adjustment of dosages and duration of administered antagonists enabled us to prevent completely the appearance of tumors in up to 60% of the mice using either of two mAbs or an Ang-binding protein with no observable adverse effects on the animals (11). Importantly, histological examination by our collaborator Dr. Marc E. Key, Vice President of Dako Corp., revealed a statistically significant decrease in the vascular density of those tumors that did develop in the Ang antagonist-treated mice. Thus, the availability of functional Ang appears critical for the establishment of these tumors suggesting a therapeutically useful approach to the treatment of Ang-dependent malignancy. We have to date demonstrated that Ang antagonists are effective in inhibiting the growth of human colon, prostate, lung, brain, fibroblast and melanocyte tumors in preclinical mouse models.

Thus, with this extensive background and experience we were well positioned to extend our antiAng therapeutic strategies to the study of human breast carcinoma. A report of our most recent work supported by the Department of Defense on breast cancer follows.

BODY (summary of progress Sept. 1, 1996 - Sept. 1, 1997)

Preclinical Mouse Models for Breast Cancer. As a major component of our Statement of Work, we proposed during the first year of support to optimize preclinical mouse models of breast carcinoma for evaluation of antitumor effects of Ang antagonists. Two models were to be used for therapy; one to assess inhibition of establishment of orthotopically implanted human MDA-MB-435 (estrogen independent) as well as MCF-7 (estrogen dependent) cell lines (termed sc prophylactic) and the second to evaluate inhibition of the development of lymph node and lung metastasis derived from orthotopically injected MDA-MB-435 cells.

We first determined that both MCF-7 and MDA-MB-435 cells secrete Ang *in vitro,* thus making them appropriate for Ang-targeted therapy. For the sc prophylactic assay, each of the two cell lines were mixed with Ang antagonists and the mixtures injected into the exposed mammary fat pad of athymic mice. Followup local injections of Ang antagonist were then administered for 24-28 days. The results presented in Figs. 1 & 2 indicate that the neutralizing antiAng mAb 26-2F is able to both delay and in a subset of mice completely prevent the establishment of both cell types in this setting. A dose-response effect of mAb 26-2F is evident and a nonspecific murine IgG (MOPC 31C) was without effect when compared to PBS-treated mice. This further reinforces our contention that antiAng therapy should be a powerful means of combating breast cancer. With these models now standardized, we will move to confirm these results, test and compare efficacy of other Ang antagonists and begin multimodal therapies combining antiAng agents with appropriate cytotoxic drugs and other antiangiogenesis molecules to determine whether increased efficacy results, as planned in our Statement of Work.

We also initiated experiments to establish a reproducible model for metastatic dissemination of orthotopically implanted MDA-MB-435 cells in athymic mice. Following published protocols we performed three experiments injecting several different tumor cell numbers but were disappointed in the number of animals that developed metastatic lesions either in regional lymph nodes or lungs. Since an assay with this low level of metastatic occurrence in controls would not be useful statistically for evaluation of the effects of antiAng inhibitors, we contacted Dr. Janet E. Price at the MD Anderson Cancer Center, the individual who originally developed this model system. In discussions with her she acknowledged that at the tumor inoculum size which she suggested in her publications, a period of up to 17 weeks was needed to achieve metastasis in 100% of control mice. She also indicated that she found it helpful to sometimes remove the primary tumor mass at about week 12 and then allowing the mice to live until week 17. If the tumor mass was removed prior to

that time, the percentage of control mice harboring metastasis dropped significantly. Following her suggestions, we were successful both in the technique of removing the primary tumor at week 12 and in subsequently observing lung metastasis at week 17. However, the lengthy period of time necessary to complete a single experiment under these conditions to us seemed excessive. In further discussions, Dr. Price indicated that she felt that achieving a critical primary tumor size rather than a long incubation period was the determining factor in producing reproducible and complete formation of metastatic lesions. As a result we decided to increase the number of tumor cells given in the initial inject to speed the growth of the primary tumor and hence resultant metastasis. In an experiment now underway, we injected 8 times the previous number of cells used. This has resulted in a tumor size approximately 4 times larger than observed with the original inoculum at the same time interval. We anticipate that with this improvement in methodology, a model assay for breast cancer metastasis will be available to us that will decrease both the expense and turnaround time for data collection. This will expedite testing of antiAng single and combinatorial therapies which is a major component of our preclinical therapeutic program.

Drug Development. Although we proposed in our original Statement of Work to postpone development of lead antiAng drugs to the second year and following, we determined that this important phase of our program could be initiated and pursued concomitant with optimization of preclinical models and testing of lead compounds. Together with our collaborators we have already made considerable progress toward developing drugs for clinical use - our ultimate goal. At present, these include:

i) *Chimerized antibody*. Since the use of mouse-derived mAbs is problematic from a clinical standpoint due to the almost certain human antimouse antibody (HAMA) response, especially for multiple treatments among which antiangiogenesis therapies should be considered, removing nonessential murine sequences of the antibody of interest and replacing them with human immunoglobulin components is necessary. During the past year we have completed construction of a chimeric antibody based on the structure of mAb 26-2F and have entered it into preclinical testing. This rapid progress has been achieved by initial critical advice from our collaborator, Dr. Richard Martinelli, at Chiron Diagnostics. The procedures followed are essentially those of Coloma et al. (12) with modifications. **Isolation of variable light (V_L) and variable heavy (V_H) chain cDNA**. Polyadenylated RNA was isolated from mAb 26-2F producing hybridoma cells using a PoliATtract System 1000 (Promega). For V_H first strand cDNA synthesis a C_H1 antisense primer was used while V_L first strand cDNA synthesis was obtained as described in the Recombinant Phage Antibody System (Mouse FcFv Module, Pharmacia Biotech). cDNAs were subsequently amplified by PCR. The PCR products were purified by agarose gel electrophoresis and eluted with the use of Magic™ PCR Preps DNA Purification System (Promega). **Subcloning and sequencing**. Each V domain cDNA was ligated into pT7Blue T-vectors (Novagen) with T4-DNA ligase. These ligation mixtures were used to transform NovaBlue competent cells (Novagen). White colonies were picked and plasmid DNA minipreps analyzed, after digestion with appropriate restriction enzymes, by agarose gel electrophoresis. Several clones, containing inserts of the expected size, were then sequenced in both directions with a Sequenase 2.0 sequencing kit (United States Biochemicals). Several independent clones were sequenced for both V_L and V_H products. The sequence of the first 16 N-terminal amino acids of each chain of mAb 26-2F, obtained by Edman degradation, showed identity to the deduced amino acid sequence obtained by cDNA sequencing. The sequences encode V_kIII and V_HIIID variable regions, as defined by Kabat et al. (13) and include the three complementarity-determining regions (CDRs) and four framework regions (FRs). **Construction of chimeric antibody genes**. V_L and V_H cDNAs were each modified at their 3'-end by eliminating the N-terminal sequence of the mouse constant region and adding a splicing signal sequence at the V_L

3'-end. This was accomplished by PCR amplification using appropriate oligonucleotide primers. For the amplification reactions, V_L and V_H cDNA fragments, obtained by EcoRV and XbaI digestion, were gel purified and PCR amplified. These were then cloned into pT7Blue T-vectors and recombinant plasmid DNAs, isolated from several colonies, were sequenced in both directions. These analyses confirmed that the expected DNA assembly was achieved. For both modified V_L and V_H domains, the cDNAs from two identical clones were excised with either EcoRV and SalI (for V_L) or with EcoRV and NheI (for V_H). The light (pAG4622) and heavy chain (pAH4604) expression vectors were constructed and kindly provided by Prof. S. L. Morrison, Dept. of Microbiology and Molecular Genetics, UCLA. Both vectors contain the genomic sequence encoding either the light (κ) or the heavy chain ($\gamma 1$) human constant domain and contain the *gpt* or *hisD* selectable markers, respectively. In order to generate chimeric murine/human antiAng antibody, V_L- and V_H-digested cDNAs were ligated into their respective expression vectors, previously digested with the appropriate enzymes. Both cDNA fragments and vectors were purified from an agarose gel prior to ligation. Moreover, for each V domain, two identical inserts, obtained from independent clones, were ligated separately into their respective vectors. The ligated products were used to transform HB101 competent cells (Promega). For both L and H chimeric chains two recombinant vectors, each derived from one of the two V region-containing clones, were isolated using the Wizard Plus Maxipreps DNA Purification System (Promega). Prior to transfection, the recombinant vectors were linearized with the PvuI isoschizomer BspCI restriction enzyme (Stratagene). Combinations of chimeric H and L chain-containing vectors were transfected simultaneously into each of two non-IgG producing mouse myeloma cell lines P3X63Ag8.653 and SP2/0 by electroporation (12). Supernatants from colonies that outgrew in the presence of selection medium were assayed by ELISA and shown to secrete intact immunoglobulins that bind to Ang. The strongest binding from each of the fusion partners were subcloned twice by limiting dilution and named P4-5 and S-13.

In order to obtain sufficient material for characterization and therapy, P4-5 and S13-1 transfectomas were injected into pristane-primed athymic mice and chimeric antibody in the resultant ascites fluid was purified by protein G-Sepharose chromatography essentially as described (9). The chimeric antibodies were shown to be as effective as the original murine mAb 26-2F at inhibiting the angiogenic activity of Ang in the chorioallantoic membrane (CAM) assay (Table1) while a nonspecific mouse IgG1κ, MOPC 31C, was without effect. We next tested the ability of the chimeric antibody to inhibit the establishment of both MDA-MB-435 and MCF-7 cells implanted orthotopically into mice. For this purpose we chose to treat with S13-1, since both chimeric constructs are identical, differing only in regard to the myeloma line used for transfection. As depicted in Figs. 1 & 2, S13-1 is indeed as effective as mAb 26-2F in its antitumor activity against both these cell types. Again, no untoward toxicities were observed. Thus, a chimeric antibody analogue of mAb 26-2F is available for subsequent therapeutic testing and eventual clinical use.

ii) *Humanized antibodies*. Since in some cases a HAMA response can still occur in patients directed against the mouse V region portions of chimeric antibodies (14), fully humanized versions of murine mAbs constructed by CDR grafting techniques are emerging for use clinically, although it is not certain at present that they will necessarily offer increased potency or decreased immunogenicity over chimeric antibodies in patients. Nevertheless, as part of our drug development program we will produce this product in addition to the chimeric antibody described above. Toward this end we have begun a collaboration with Dr. K. R. Acharya at the University of Bath, UK, an expert in x-ray crystallographic techniques and protein modeling. Dr. Acharya, in continuing collaboration with this Center has solved the crystal structures of both bovine (15) and human Ang (16) to 1.5 and 2.0 Å, respectively. Dr. Martinelli will also consult with us during this process. As a step toward humanization, Fab fragments of mAb 26-2F produced by us have been supplied to Dr.

Acharya and crystals of the Fab-human Ang complex have recently been successfully produced. Encouragingly, Dr. Acharya working at the synchrotron at Daresbury, UK has shown that the crystals do indeed diffract and he has collected a complete data set at 3.1 Å resolution. As this resolution is not sufficient to model optimized humanized antibodies, further studies to increase sensitivity are underway. It is anticipated that within the year the 3-dimensional structural analysis of this complex will be completed leading the way toward the design of humanized antibodies.

iii) *Small Molecule Inhibitors.* Previous studies have demonstrated that the ribonucleolytic activity of Ang, although extremely weak with conventional substrates, is nonetheless essential for the biological action of the protein. Therefore, molecules that are potent inhibitors of this activity might have considerable potential as anti-cancer agents. The best small molecule inhibitors of Ang now available are 5'-diphosphoadenosine 2'-monophosphate, (ppA-2'-p) and its 2'-deoxyuridine derivative (dUppA-2'-p). Although neither ppA-2'-p nor dUppA-2'-p binds sufficiently tightly to be useful as a drug, their interactions are strong enough to allow structural studies to be performed. Full sets of NMR data on both complexes have now been collected by our collaborator Dr. Feng Ni at the National Research Council of Canada and calculations of the three-dimensional structures should be completed within several months. Another collaborator, Dr. Ravi Acharya, has also made major recent progress toward the determination of crystal structures of inhibitor complexes. These NMR and X-ray structures will provide a basis for initiating the proposed rational design efforts. Computer modeling will be performed to identify alterations in inhibitor structure that are predicted to improve binding affinity significantly. These changes will presumably involve attachment of additional substituents or replacement of existing components. In both cases the aim is to increase the number of contacts between the inhibitor and its target, and to make the inhibitor structure more complementary to that of the Ang active site. Models of complexes with the new compounds will be generated and subjected to energy minimization. The most promising compounds identified will be synthesized and assayed for their effectiveness as inhibitors of the enzymatic activity of Ang against polynucleotide and dinucleotide substrates. Any compounds that exhibit substantially increased potency will be tested as inhibitors of the angiogenic activity of Ang, and as antagonists of breast tumor formation in mice. If any further improvement in binding strength is necessary, the most effective of the new inhibitors will be examined in a second round of structural studies by NMR or X-ray crystallography, and the structures obtained will be used for further modeling and inhibitor design. These additional structures are necessary since modified inhibitors often do not bind in precisely the manner predicted. The resultant "third-generation" compounds will be synthesized and their effects on Ang function will be evaluated.

iv) Antisense Drugs. Although not a part of our original proposal, we are considering additional strategies to inhibit the tumor-associated actions of Ang which do not necessarily require direct binding to the Ang protein. One of these includes interference with Ang *gene expression* using antisense technologies. **Background**. Enormous advances have been made over the last several years in the development of synthetic antisense oligodeoxynucleotides (ODNs) for therapeutic use. As therapeutics they possess two major requirements for successful drug design - specificity and affinity. Specificity is achieved by selectively targeting particular RNA sequences by exploiting Watson-Crick base pairing resulting in interference with the translational process. This approach allows for rapid identification of lead compounds based on knowledge of a relevant target gene's sequence. Recently, improvements have been made in increasing both the stability and affinity of these compounds. First-generation phosphorothioate analogues of ODNs, in which nonbridging phosphoryl oxygens in the backbone of DNA are substituted with sulfur - [S]ODNs - have substantially increased nuclease stability. [S]ODNs possess good biological activity, pharmacology and safety *in vivo* (17). Importantly, the costs for preparing these agents is rapidly

decreasing (18). Several biotechnology companies (e.g., Isis, Hybridon) have embraced this strategy and [S]ODNs are now in clinical trials for treatment of cancers and viral infections (19). A milestone for the technology was reached in early 1997 when Isis presented the results of a phase II clinical trial in which systemic administration of an antisense [S]ODN showed considerable *efficacy* in addition to safety for the treatment of Crohn's disease patients (20). As a result of the considerable promise of antisense therapeutics, we began a program to evaluate use of these types of drugs that target Ang for the treatment of breast cancer. **Results to date**. Successful inhibition of specific gene function has been achieved by targeting various portions of a specific mRNA sequence including the AUG translational initiation codon, 5' transcriptional start site, 3' termination codon as well as sequences in both the 5' and 3' untranslated regions. Experience to date indicates that considerable success has been obtained by targeting the first of these regions. We have previously determined the sequence of the entire cDNA as well as 5'- and 3'-flanking regions for Ang (21) . As a consequence, an antisense 18-mer [S]ODN was custom synthesized based on sequences encompassing the AUG initiation codon region of the *Ang* gene and named JF2S. Its corresponding sense control, termed JF1S, was also synthesized. Initially, experiments were performed *in vitro* aimed at determining whether this antisense reagent was effective in inhibiting the synthesis of Ang by MDA-MB-435 cells and what effect this *ex vivo* treatment would have on the subsequent growth of these cells in athymic animals. Efficient transfection of ODNs *in vitro* requires the presence of a cationic lipid; one of which, lipofectin, is commercially available. In these experiments cultured MDA-MB-435 cells were treated twice over 48 h with either lipofectin plus JF2S or its sense JF1S control or lipofectin alone. These *ex vivo*-treated cells were then harvested (conditioned medium was saved for determination of Ang concentrations) and injected sc into athymic mice (5 or 7 mice/group). At a time point when tumors from untreated cells had grown to a measurable size, animals were sacrificed and tumors, where present, were weighed and photographed. As depicted in Fig. 3, *ex vivo* treatment of breast tumor cells with JF2S inhibits Ang expression *in vitro* which translates into a dramatic decrease in the average size (by weight) of the resulting tumors *in vivo* when compared to those derived from control lipofectin- or sense-treated cells. In fact, a tumor did not form at all in 1 mouse injected with JF2S. An actual photograph of the excised tumors from this experiment is shown in Fig. 4. This again indicates that Ang is indeed critical for the growth of breast tumor cells *in vivo* and, as a result, therapy experiments using JF2S for the treatment of breast carcinoma were begun.

In the first set of experiments the sc prophylactic model of tumor growth described above was used. As depicted in Figs. 5 & 6, JF2S was able to delay and prevent the establishment of both MDA-MB-435 and MCF-7 cells at a level comparable to that achieved with mAb 26-2F (see Figs. 1 & 2). The sense control JF1S was without effect and no toxicities in animals were observed. Additionally, we determined that JF2S is not directly cytotoxic to either of these breast cancer cell lines *in vitro* suggesting that the therapeutic effects observed *in vivo* are mediated by an antiangiogenic process via inhibition of tumor-associated Ang expression. A portion of our initial findings on Ang-directed antisense therapy for breast cancer was presented at the 1997 annual meeting of the American Association for Cancer Research (22). Thus, based on these data we will continue to evaluate antiAng antisense drugs alone and in combination with other agents for the treatment of breast carcinoma and its metastasis.

Immunohistochemical Analysis of Ang Expression in Clinical Breast Carcinoma. Together with our collaborator, Dr. Marc Key, Vice President of Laboratory Operations, Dako Corp., we have begun a study to determine the incidence and location of Ang expression in clinical breast cancer samples. This information will eventually be critical in determining the patient population for which antiAng therapy should be considered. In initial studies, Dr. Key has examined breast tumor tissue

maintained in the human tissue bank at Dako Corp. Ten separate breast carcinomas obtained during surgery were fixed in formalin and embedded in paraffin. Immunohistochemical analysis was performed on cut sections utilizing the antiAng mAb 26-2F and the Catalyzed Signal Amplification (CSA) detection method developed by Dako. Using this procedure, all ten specimens were positive for Ang staining. Strongest staining was found in the stroma, particularly surrounding nests of tumor cells-an observation consistent with tumor cell secretion of Ang protein. Within well-differentiated nests of ductal carcinoma, Ang was predominantly localized to the cytoplasm of the outer cells (myoepithelium and adjacent epithelial cells). In poorly differentiated carcinomas, tumor cells frequently displayed Ang staining in the nucleus of 10-30% of the cells. Both inflammatory and residual tumor cells in necrotic areas were strongly positive while in hemorrhagic areas extracellular staining was frequently observed.

Thus, we have already determined that Ang is indeed present in clinical specimens of breast tumor tissue. These studies will be extended to include a wide range of breast tumor histologic types, grades, etc., along with corresponding normal breast tissue to gain further insights into incidence and underlying mechanism(s) of Ang production by breast carcinoma cells.

CONCLUSIONS

During the first year of Department of Defense support we have achieved and expanded upon our original proposal as defined in the Statement of Work. Preclinical models are now in place for testing the effects of Ang antagonists on the establishment and metastatic growth of human breast cancer. Indeed, we have already shown that an antiAng neutralizing monoclonal antibody is effective in inhibiting the establishment of both hormone dependent and independent breast carcinoma cell lines. In addition, development of drugs toward eventual clinical use is proceeding. We have already constructed a chimeric antibody analogue that is as effective as its murine counterpart in inhibiting tumor cell growth. Small molecule inhibitors are also under development. Importantly, we have shown that Ang is present in clinical samples of breast tumor tissue supporting our contention that antiAng therapies will be effective in treating patients with this disease. Finally, we have begun a major initiative employing Ang antisense agents as therapeutic alternatives. Thus, inhibitors of the activities or expression of Ang offer potential powerful tools for the treatment of carcinoma of the breast and its metastasis.

REFERENCES

1. Parker, S.L., Tong, T., Bolden, S., and Wingo, P.A. Cancer Statistics. *CA Cancer J. Clin.* **47**, 5-27, 1997.
2. Folkman, J. What is the Evidence that Tumors are Angiogenesis Dependent? *J. Natl. Cancer Inst.* **82**, 4-6, 1989.
3. Blood, C.H., and Zetter, B.R. Tumor Interactions with the Vasculature: Angiogenesis and Tumor Metastasis. *Biochim. Biophys. Acta* **1032**, 89-118, 1990.
4. Taki, T., Ohnishi, T., Arita, N., Hiraga, S., Saitoh, Y., Isumoto, S., Mori, K., and Hayakawa, T. Anti-Proliferative Effects of TNP-470 on human malignant glioma *in vivo*: potent inhibition of Tumor Angiogenesis. *J. Neuro-Oncol.*, **19**, 251-258, 1994.
5. Borgström, P., Hillan, K.J., Sriramarao, P., and Ferrara, N. Complete Inhibition of Angiogenesis and Growth of Microtumors by Anti-Vascular Endothelial Growth Factor Neutralyzing Antibody: Novel Concepts of Angiostatic Therapy from Intravital Videomicroscopy. *Cancer Res.* **56**, 4032-4039, 1996.
6. Weidner, N, Folkman, J., Pozza, F., Bevilacqua, P., Albred, E.N., Moore, D.H., Meli, S., and Gasparini, G. Tumor Angiogenesis: A New Significant and Independent Prognostic Indicator in Early Stage Breast Carcinoma. *J. Natl. Cancer Inst.* **84**, 1875-1887, 1992.
7. Fett, J.W., Strydom, D.J., Lobb, R.R., Alderman, E.M., Bethune, J.L., Riordan, J.F., and Vallee, B.L. Isolation and Characterization of an Angiogenic Protein from Human Carcinoma Cells. *Biochemistry* **24**, 5480-5486.
8. Acharya, K.R., Shapiro, R., Allen, S.C., Riordan, J.F., and Vallee, B.L. Crystal Structure of Human Angiogenin Reveals the Structural Basis for its Functional Divergence from Ribonuclease. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2915-2919, 1994.
9. Fett, J.W., Olson, K.A., and Rybak, S.M. A Monoclonal Antibody to Human Angiogenin. Inhibition of Ribonucleolytic and Angiogenic Activities and Localization of the Antigenic Epitope. *Biochemistry* **33**, 5421-5427, 1994.
10. Olson, K.A., French, T.C., Vallee, B.L., and Fett, J.W. A Monoclonal Antibody to Human Angiogenin Suppresses Tumor Growth in Athymic Mice. *Cancer Res.* **54**, 4576-4579, 1994.
11. Olson, K.A., Fett, J.W., French, T.C., Key, M.E., and Vallee, B.L. Angiogenin Antagonists Prevent Tumor Growth *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 442-446, 1995.
12. Coloma, M.J., Hastings, A., Wims, L.A., and Morrison, S.L. Novel Vectors for the Expression of Antibody Moleculoes using Variable Regions Generated by Polymerase Chain Reaction. *J. Immunol. Meth.* **152**, 89-104, 1992.
13. Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., and Foeller, C. Sequences of Proteins of Immunological Interest. U.S. Dept. Health and Human Services, NIH Publication No. 91-3242, 5th Edition, 1991.
14. Baker, T.S., Bose, C.C., Caskey-Finney, H.M., King, D.J., Lawson, A.D.G., Lyons, A., Mountain, A., Owens, R.J., Rolfe, M.R., Sehdev, M., Yarrington, G.T.R., and Adair, J.R. Humanization of an AntiMucin Antibody for Breast and Ovarian Cancer Therapy. In Ceriani, R.L. (ed). *Antigen and Antibody Molecular Engineering in Breast Cancer Diagnosis and Treatment*, Plenum Press, 61-82, 1994.
15. Acharya, K.R., Shapiro, R., Riordan, J.F., and Vallee, B.L. Crystal Structure of Bovine Angiogenin at 1.5 Å Resolution. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2949-2953.
16. Russo, N., Acharya, K.R., Vallee, B.L., and Shapiro, R. A Combined Kinetic and Modeling Study of the Catalytic Center Subsites of Human Angiogenin. *Proc. Natl. Acad. Sci. U.S.A.*

93, 804-808, 1996.

17. *Antisense Therapeutics*. Agrawal, S. (ed). Humana Press, 1996.
18. Hodgson, J. Antisense Companies Reach Reality. *Nature Biotech.* **14**, 815-816, 1996.
19. Larkin, M. Genset and Synthelabo Achieve Oligo-Related Milestone. *GEN* **17** (No. 2), 1, 1997.
20. Isis Pharmaceuticals Demonstrates Efficacy in Crohn's Disease with its Antisense Drug. *GEN*, **17** (No. 5), 1, 1997.
21. Kurachi, K., Davie, E.W., Strydom, D.J., Riordan, J.F., and Vallee, B.L. *Biochemistry* **24**, 5486-5494, 1985.
22. Fett, J.W., and Olson, K.A. Anti-Angiogenin Gene Therapy for Solid Tumors. *Proc. Am. Assoc. Cancer Res.* **38**, 65A, 1997.

APPENDICES (illustrations)

Table 1: Effect of mAb 26-2F, S13-1 and P4-5 on the Activity of Human Ang in the CAM Assay[a]

group	Ang	mAb 26-2F	S13-1	P4-5	MOPC 31C	assay results[b]	p[c]	status
I	+	-	-	-	-	25/45 (56)	0.0009	active
II	+	+	-	-	-	10/45(22)	0.9556	inactive
III	+	-	+	-	-	11/46(24)	0.8038	inactive
IV	+	-	-	+	-	11/45(24)	0.7594	inactive
V	+	-	-	-	+	26/45(58)	0.0004	active
VI	-	+	-	-	-	9/42(21)	0.9718	inactive
VII	-	-	+	-	-	7/45(16)	0.4492	inactive
VIII	-	-	-	+	-	13/42(31)	0.3258	inactive
IX	-	-	-	-	+	15/45(33)	0.2154	inactive

[a]Combined data represent 3 sets of assays. Each individual assay employed between 15 and 19 eggs. [b]These are expressed as the ratio of positive to total surviving eggs. The percentage of positive eggs is given in parentheses. [c]Significance was calculated from χ^2 values of data recorded at 48 ± 2 h based on comparison with water controls tested simultaneously (10 positive eggs/46 total surviving eggs, 22% positive). To be designated active samples must have a value of $p < 0.05$. Amount applied per egg is10 ng of Ang and 100 ng of IgGs.

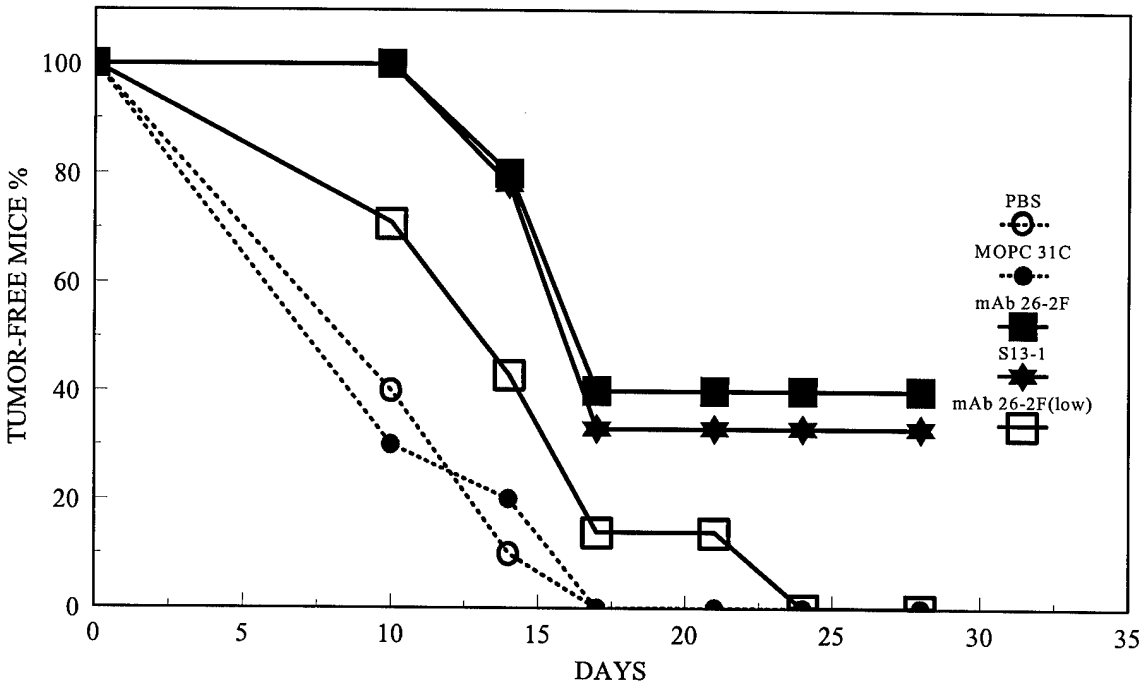

Figure 1. Prevention of MDA-MB-435 tumor growth in athymic mice by treatment with mAb 26-2F or its chimeric analog, S13-1. Mixtures of cells (5 x 10^5/mouse) with either PBS (dashed line with open circles), MOPC 31C (nonspecific IgG control, 240 μg/dose, dashed line with closed circles), mAb 26-2F (240 μg/dose, solid line with closed squares), S13-1 (240 μg/dose, solid line with stars) or a lower dose of mAb 26-2F (120 μg/dose, solid line with open squares) were injected into the surgically exposed mammary fat pad on day 0. Mice were then treated sc with one half the day 0 dose of the same material 6 times per week until day 28. n=10 for all groups except for the S13-1 group (n=9) and the mAb 26-2F low dose group (n=7).

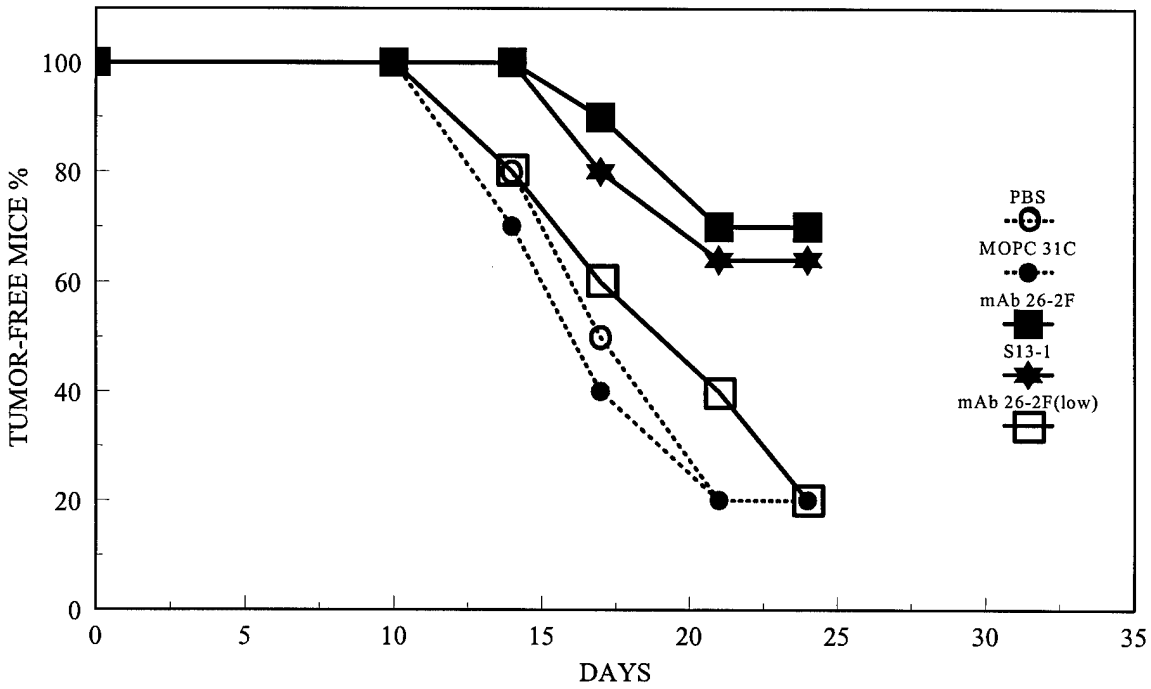

Figure 2. Prevention of MCF-7 tumor growth in athymic mice by treatment with mAb 26-2F or its chimeric analog, S13-1. Mixtures of cells (1 x 10^6/mouse) with either PBS (dashed line with open circles), MOPC 31C (nonspecific IgG control, 240 μg/dose, dashed line with closed circles), mAb 26-2F (240 μg/dose, solid line with closed squares), S13-1 (240 μg/dose, solid line with stars) or a lower dose of mAb 26-2F (120 μg/dose, solid line with open squares) were injected into the surgically exposed mammary fat pad on day 0. A pellet containing 17 β-estradiol (0.72 mg/pellet, 60-day release) as the source of exogenous estrogen necessary for the growth of this cell line in athymic mice was placed sc through the same incision 1cm from the cell injection site. Mice were then treated sc with the same dose of material 6 times per week until day 24. n=10 for all groups except for the S13-1 group (n=11) and the mAb 26-2F low dose group (n=5).

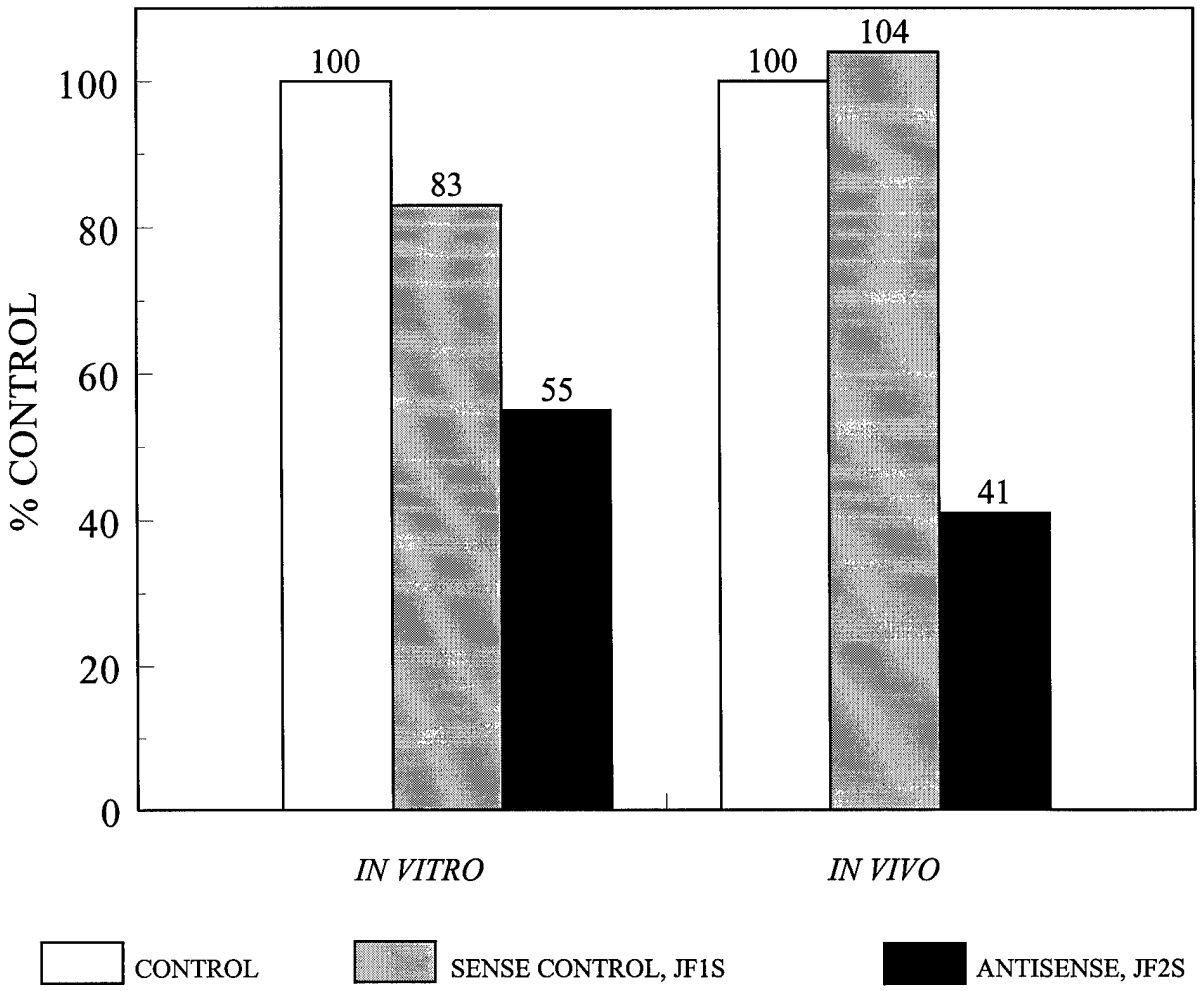

Figure 3. Treatment of MDA-MB-435 breast tumor cells *in vitro* with Ang antisense JF2S or control sense JF1S - inhibition of Ang production *in vitro* and tumor size *in vivo*. MDA-MB-435 cells grown in 100-mm diameter tissue culture dishes were treated with lipofectin alone (5 μg/ml, white) as control, lipofectin plus Ang sense control JF1S (1μM, gray) or lipofectin plus Ang antisense JF2S (1μM, black). The treatments were repeated 24 hr later. The following day supernatants were collected and the Ang concentrations were determined by ELISA. The tumor cells were subsequently trypsinized, washed with HBSS and injected into the surgically exposed mammary fat pad of athymic mice (5 x 10^5 cells/mouse). After 19 days the mice were sacrificed and the tumors excised, weighed and photographed. The amount of Ang per 10^6 cells for each group in percent compared to that of the lipofectin control group (100%) is depicted (*IN VITRO*). The average tumor size by weight for each group compared to that of the lipofectin control group (100%) is shown *(IN VIVO)*. n=5 for all groups except the antisense-treated group for which n=7.

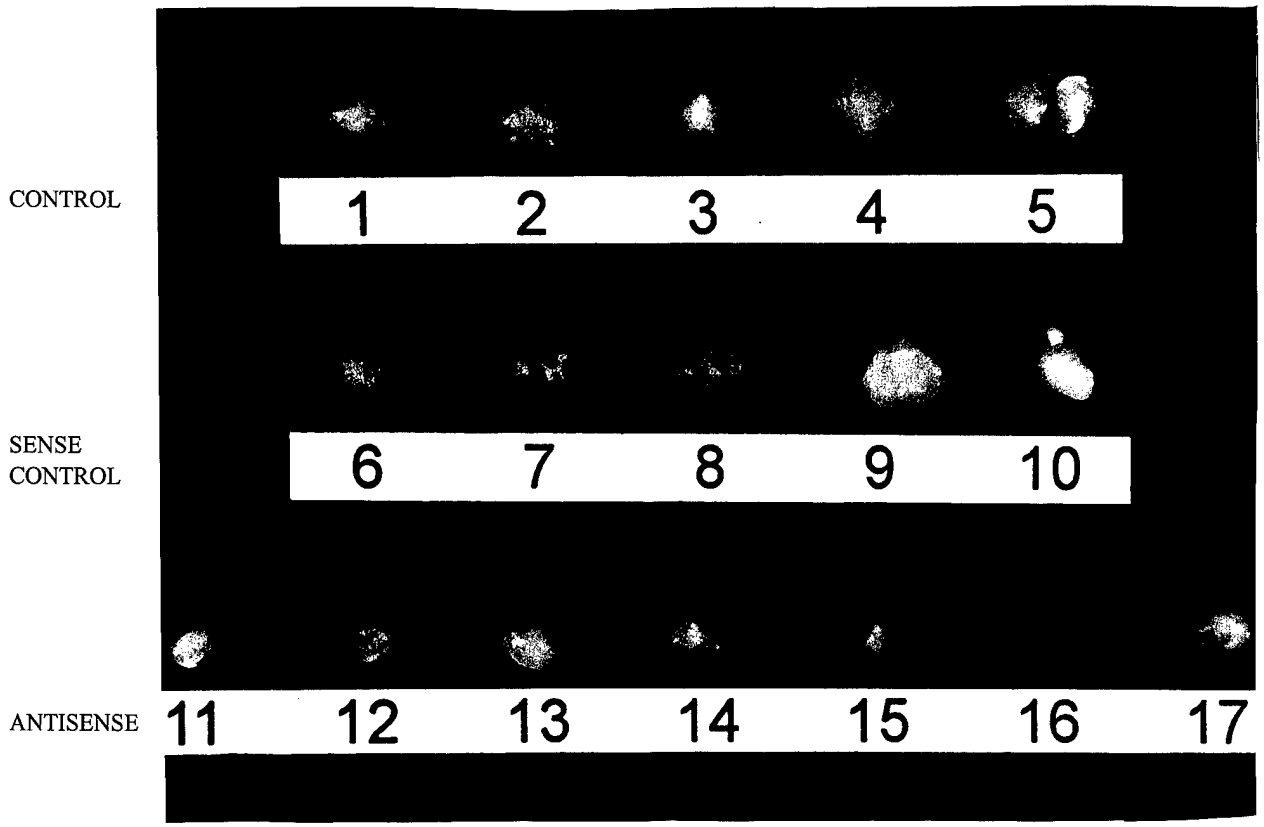

Figure 4. Treatment of MDA-MB-435 breast tumor cells *in vitro* with Ang antisense JF2S or control sense JF1S. Photograph of excised tumors from the experiment shown in Figure 3. The average tumor weights for each of the treatment groups are reported in that figure (*IN VIVO*).

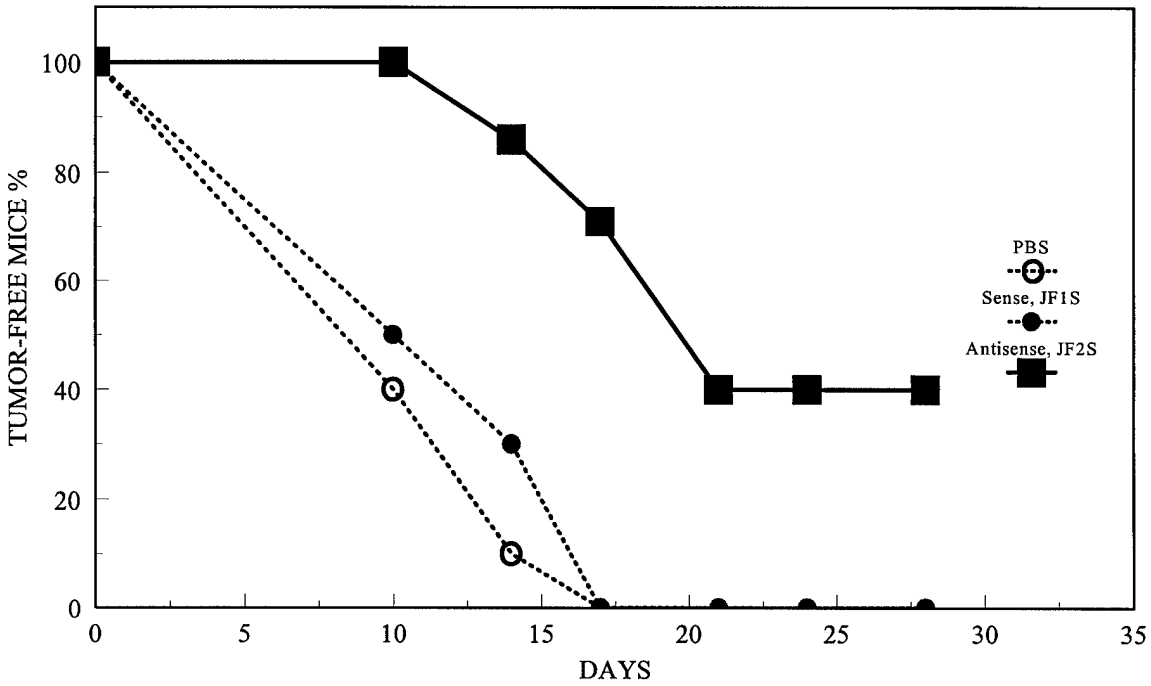

Figure 5. Prevention of MDA-MB-435 tumor growth in athymic mice by treatment with Ang antisense JF2S. Mixtures of cells (5 x 10^5/mouse) with either PBS (dashed line with open circles), Ang sense control JF1S (200 μg/dose, dashed line with closed circles) or Ang antisense JF2S (200 μg/dose, solid line with closed squares) were injected into the surgically exposed mammary fat pad on day 0. Mice were then treated sc with one half the day 0 dose of the same material 6 times per week until day 28. n=10 for PBS- and JF1S-treated groups and n=7 for the JF2S-treated group.

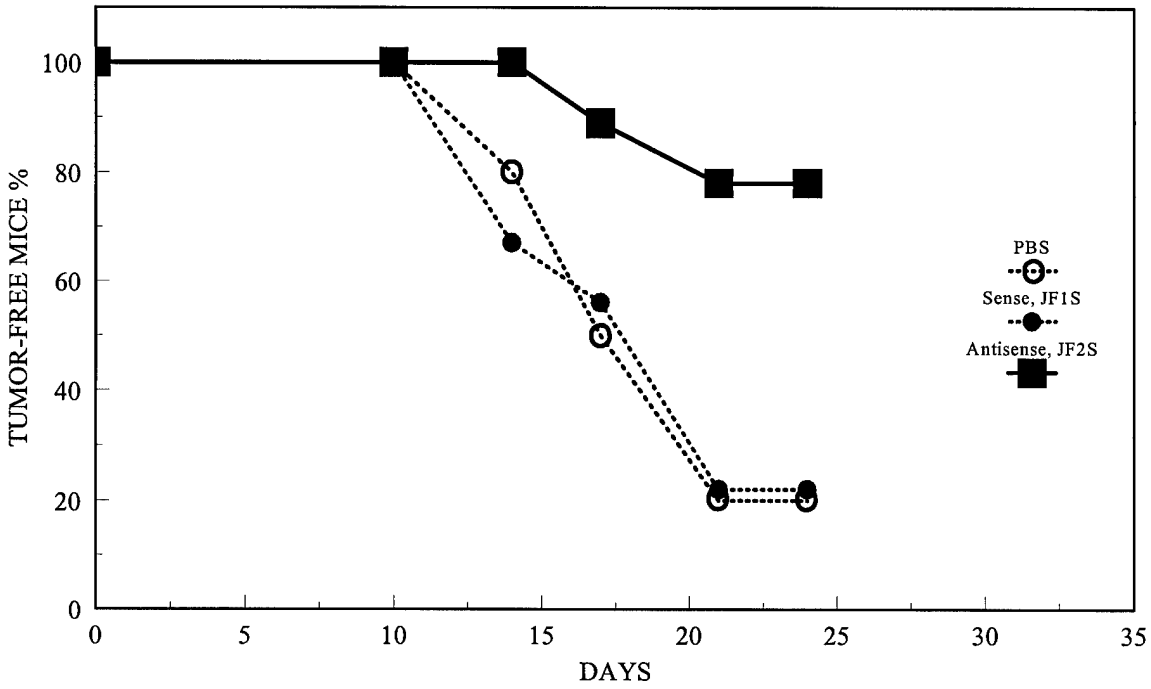

Figure 6. Prevention of MCF-7 tumor growth in athymic mice by treatment with Ang antisense JF2S. Mixtures of cells (1 x 10^6/mouse) with either PBS (dashed line with open circles), Ang sense control JF1S (200 μg/dose, dashed line with closed circles) or Ang antisense JF2S (200 μg/dose, solid line with closed squares) were injected into the surgically exposed mammary fat pad on day 0. A pellet containing 17 β-estradiol (0.72 mg/pellet, 60-day release) as the source of exogenous estrogen necessary for the growth of this cell line in athymic mice was placed sc through the same incision 1 cm from the cell injection site. Mice were then treated sc with the same dose of material 6 times per week until day 24. n=10 for PBS-treated group and n=9 for the JF1S- and JF2S-treated groups.